Remarks

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendment, claims 7-9, 14-16 and 35-40 are pending in the application, with claim 7 being the sole independent claim. Claims 7, 14 and 36 are sought to be amended. New claims 37-40 are sought to be added. These changes are believed to introduce no new matter, and their entry is respectfully requested.

Based on the above amendment and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

I. Support for Amended and New Claims

Support for the amended and new claims can be found throughout the specification. Support for amended claims 7 and 36 can be found, for example, at page 17, line 17 through page 18, line 9, and at page 46, lines 4-26. Support for amended claim 14 can be found, for example, at page 21, lines 3-20. Support for new claim 37 can be found, for example, at page 20, line 26 through page 21, line 2, and at page 21, lines 3-20. Support for new claim 38 can be found, for example, at page 18, lines 15-18, at page 20, lines 1-3, and in original claim 7. Support for new claims 39 and 40 can be found, for example, at page 13, lines 7-12.

II. Claim Objection

Claim 7 was objected to for allegedly reciting the phrase "all of whose germ and somatic cell line." *See* Paper No. 14, page 2. Applicants note that claim 7 does not contain the phrase "all of whose germ and somatic *cell line*." Rather, claim 7 recites: "all of whose germ and somatic cells. . ." *See* original claim 7 and Applicants' Amendment and Reply filed August 8, 2002 (hereinafter "the August 8, 2002 Response") at pages 3 and 34. Applicants also note that claim 7 has been amended to recite "A transgenic non-human animal whose germ and somatic cells. . ." Therefore, the objection to claim 7 has been accommodated and should be withdrawn.

III. Claim Rejections Under 35 U.S.C. § 112, First Paragraph

A. Written Description

Claims 7, 9, 14, 16, 35 and 36 were rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. *See* Paper No. 14, pages 2-3. Applicants respectfully traverse this rejection.

The rejection appears to be based, at least in part, on the recitation of the phrase "wherein said DNA molecule codes for a protein that has an activity of AD7c-NTP when *expressed* in neuronal cells." According to the Examiner:

> the as-filed specification provides sufficient description for a genus of a transgenic non-human animal whose genome comprises a DNA molecule of SEQ ID NO: 1 or a DNA

> molecule which is 90% homologous thereto, wherein said DNA molecule is *over-expressed* in neuronal cells that results in neuritic sprouting, nerve cell death, nerve cell degeneration, neurofibrillary tangles, and/or irregular swollen neurites. . .
>
> [T]he as-filed specification does not provide sufficient description of a representative number of species of transgenic non-human animal whose genome comprises a DNA molecule which is 90% homologous to SEQ ID NO: 1 and wherein said DNA molecule codes for a protein that has an activity of AD7c-NTP when expressed in neuronal cells.

Paper No. 14, pages 7-8 (emphasis in original).

Applicants note that claim 7 has been amended to recite ". . .wherein said DNA molecule codes for a protein that has an activity of AD7c-NTP when *over*-expressed in neuronal cells." (Emphasis added). The purpose of this amendment is to make explicit an implicit feature of the invention. Thus, the rejection, insofar as it relates to the recitation of the phrase "wherein said DNA molecule codes for a protein that has an activity of AD7c-NTP when *expressed* in neuronal cells," has been fully accommodated.

The rejection is also based on the contention that:

> the as-filed specification does not provide sufficient description of a phenotype of a transgenic animal comprising a nucleotide sequence that is 90% homologous to SEQ ID NO: 1 if a phenotype comprising neuritic sprouting, nerve cell death, nerve cell degeneration, neurofibrillary tangles, and/or irregular swollen neurites in neuronal cells in neuronal cells [sic] is not observed in the transgenic animal.

Paper No. 14, page 8.

Applicants note that present claims 7-9, 14-16, 35 and 36 do not specify any particular phenotype that the transgenic animals of the invention must possess. The only specified features of the transgenic animals encompassed by these claims are: (1) that their germ and somatic cells comprise the DNA molecule of SEQ ID NO:1 or a DNA molecule

which is at least 90% homologous thereto; and (2) that the DNA molecule is over-expressed in one or more cells of the transgenic animal. Section 112, first paragraph, requires only that the *claimed invention* be adequately described in the specification. *See Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1560, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991). Thus, in terms of satisfying the written description requirement of 35 USC § 112, first paragraph, it is not necessary that a skilled artisan be able to envision any particular phenotype of the transgenic animals encompassed by or used in the practice of claims 7-9, 14-16, 35 and 36.

New claims 37 and 38 are directed to screening methods that comprise administering a candidate drug to the transgenic animal of claim 7, wherein the transgenic animal exhibits at least one of neuritic sprouting, nerve cell death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons. The specification describes methods for producing such transgenic animals using techniques that were known in the art. *See* specification at page 20, lines 1-25. The specification also describes methods for analyzing transgenic animals for the recited neuronal abnormalities. *See* specification at page 20, line 26 through page 21, line 2. The specification describes methods that comprise the use of the transgenic animals to screen for candidate drugs that are potentially useful for the treatment or prevention of Alzheimer's disease, neuroectodermal tumors, malignant astrocytomas and glioblastomas. *See* specification at page 21, lines 3-21. The specification further describes and illustrates the neuronal abnormalities that are caused by over-expressing AD7c-NTP in neuronal cells. *See* specification at page 46, lines 4-26 and Figs. 6A-6G. Thus, the methods of new claims 37 and 38 are adequately described in the specification.

A person of ordinary skill in the art would have recognized that Applicants were in possession of the transgenic animals and methods encompassed by the present claims.

Therefore, the written description requirements of 35 U.S.C. § 112, first paragraph, are fully satisfied. Applicants therefore respectfully request that the rejection of claims 7, 9, 14, 16, 35 and 36 under 35 U.S.C. § 112, first paragraph, for insufficient written description, be reconsidered and withdrawn.

B. Enablement

Claims 7-9, 14-16, 35 and 36 were rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected to make and/or use the invention. *See* Paper No. 14, page 8. Applicants respectfully traverse this rejection.

1. The Claimed Invention is Fully Enabled

In order to satisfy the enablement requirement of 35 USC § 112, first paragraph, the claimed invention must be enabled so that any person skilled in the art can make and use the invention without undue experimentation. *See In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). Since it would require only routine experimentation for a person of ordinary skill in the art to make and use the transgenic non-human animals of claims 7-9, 35 and 36, and to practice the methods of claims 14-16 (and those of new claims 37 and 38), the enablement requirement of 35 U.S.C. § 112, first paragraph, is fully satisfied.

(a) Claims Directed to Transgenic Non-Human Animals are Fully Enabled

Claims 7-9, 35 and 36, as currently presented, are directed to transgenic non-human animals, all of whose germ and somatic cells comprise the DNA molecule of SEQ ID NO:1 or a DNA molecule which is at least 90% homologous thereto, wherein said DNA molecule is expressed in one or more cells of said transgenic animal, and wherein said DNA molecule codes for a protein that has an activity of AD7c-NTP when over-expressed in neuronal cells. A person of ordinary skill in the art would have been able to make and use the transgenic non-human animals encompassed by these claims using routine methods in the art.

(i) Obtaining a DNA Molecule of SEQ ID NO:1 or a DNA Molecule Which is at Least 90% Homologous Thereto

A person of ordinary skill in the art would have been able to obtain the DNA molecules that are used to produce the transgenic animals of the invention. More specifically, a person of ordinary skill in the art, in view of the present specification, would have been able to obtain a DNA molecule of SEQ ID NO:1 or a DNA molecule which is at least 90% homologous thereto, wherein the DNA molecule codes for a protein that has an activity of AD7c-NTP when over-expressed in neuronal cells, using only routine methods in the art.

The specification provides exemplary methods for obtaining DNA molecules which are at least 90% homologous to SEQ ID NO:1. Such methods involve the isolation of DNA molecules from cDNA libraries by hybridization under stringent conditions to the DNA molecule of SEQ ID NO:1. *See* specification at page 19, lines 3-15. Additional methods

for obtaining DNA molecules that are at least 90% homologous to SEQ ID NO:1 include the use of directed and random mutagenesis techniques. Such methods were well known to those of ordinary skill in the art at the time of the invention. *See, e.g.*, Sambrook *et al.*, "Creating Many Mutations in a Defined Segment of DNA," in *Molecular Cloning, A Laboratory Manual*, Sambrook *et al.*, eds., Cold Spring Harbor Laboratory Press, pp. 15.95-15.108 (1989) (copy attached herewith as Exhibit 1).

Once obtained, DNA molecules that are at least 90% homologous to SEQ ID NO:1 could have easily been tested for the ability to encode a protein having an activity of AD7c-NTP. The specification describes various methods for assaying for AD7c-NTP activity. For example, transgenic animals can be made that over-express AD7c-NTP in neuronal cells, and, once obtained, the transgenic animals may be analyzed for evidence of neuronal or neuritic abnormalities associated with Alzheimer's disease, neuroectodermal tumors, malignant astrocytomas and glioblastomas. *See* specification at page 20, lines 1-29. (The ability of a person of ordinary skill in the art to produce transgenic animals whose germ and somatic cells comprise the DNA molecule of SEQ ID NO:1 or a DNA molecule which is at least 90% homologous thereto is discussed in more detail below).

Additionally, *in vitro* methods can be used to test for AD7c-NTP activity. For example, the specification exemplifies an assay involving the overexpression of AD7c-NTP in neuronal cells and the subsequent analysis for cellular characteristics of Alzheimer's disease, including apoptosis and neuritic sprouting. *See* specification at page 46, lines 4-26. Thus, the full range of DNA molecules that are included within the transgenic animals of the invention could have been easily made and analyzed by persons of ordinary skill in the art using only routine methods and experimentation.

(ii) Creating Transgenic Non-Human Animals

A person of ordinary skill in the art, using routine methods in view of the present specification, would also have been able to create transgenic non-human animals, all of whose germ and somatic cells comprise the DNA molecule of SEQ ID NO:1 or a DNA molecule which is at least 90% homologous thereto, and wherein the DNA molecule is over-expressed in one or more cells of the transgenic animal.

The enablement rejection appears to be based, in large part, on the Examiner's contention that producing transgenic animals that express a particular phenotype is unpredictable. *See*, *e.g.*, Paper No. 14, page 15 ("the art of transgenic is not [a] predictable art with respect to transgene behavior and the resulting phenotype.") Only new claims 37 and 38, however, specify that the transgenic animals used in the claimed methods exhibit any particular attribute, *i.e.*, at least one of neuritic sprouting, nerve cell death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons. Applicants submit that a person of ordinary skill in the art, in view of the present specification, would have been able to produce transgenic animals having one or more of the above-described attributes using only routine experimentation.

The specification describes exemplary methods for obtaining transgenic animals including, *e.g.*, injecting a DNA construct into a fertilized egg which is allowed to develop into an adult animal, and generating transgenic animals with embryonic stem cell technology. *See* specification at page 20, lines 3-17. The specification also provides several references which describe additional methods of preparing transgenic animals. *See* specification at page 20, lines 18-25. For example, the specification at page 20, line 19, cites

U.S. Patent No. 5,602,299 (hereinafter "Lazzarini") (incorporated by reference in the specification in its entirety). Lazzarini states:

> Any technique known in the art may be used to introduce the transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to pronuclear microinjection; retrovirus mediated gene transfer into germ lines; gene targeting in embryonic stem cells; electroporation of embryos; and sperm-mediated gene transfer; etc.

See U.S. Patent No. 5,602,299 at column 13, lines 2-12 (internal citations omitted).

In addition to the teachings of the specification, the knowledge available in the art would have provided additional guidance for the production of the transgenic animals of the invention. An Applicant is not limited to the confines of the specification to provide the necessary information to enable an invention. *See In re Howarth*, 654 F.2d 103, 105-6, 210 USPQ 689, 692 (CCPA 1981). An Applicant need not supply information that is well known in the art. *See Genentech, Inc. v. Novo Nordisk*, 108 F.3d 1361, 1366, 42 USPQ2d 1001, 1005 (Fed. Cir. 1997); *Howarth*, 654 F.2d at 105-6, 210 USPQ at 692; *see also In re Brebner*, 455 F.2d 1402, 173 USPQ 169 (CCPA 1972) (finding a disclosure enabling because the procedure for making the starting material, although not disclosed, would have been known to one of ordinary skill in the art as evidenced by a Canadian patent). "'That which is common and well known is as if it were written out in the patent and delineated in the drawings.'" *Howarth*, 654 F.2d at 106, 210 USPQ at 692 (quoting *Webster Loom Co. v. Higgins* et al., 105 U.S. (15 Otto.) 580, 586 (1881)). Moreover, one of ordinary skill in the art is deemed to know not only what is considered well known in the art but also where to search for any needed starting materials. *Id.*

Applicants, in their previous response, presented additional examples from the scientific literature (available as of the effective filing date of the present application) that demonstrate the successful production of transgenic animals that exhibited particular phenotypes indicative of the transgene. *See* the August 8, 2002 Response at pages 31-32. These references (submitted with the August 8, 2002 Response as Exhibits B-E) describe: (i) the production of transgenic rats that expressed the human HLA-B27 gene and that exhibited a spontaneously arising disease that "showed a striking clinical and histologic similarity to B27-associated disease in humans," *see* Hammer, R.E. *et al., Cell 63*:1099-1112 (1990), *see* especially page 1108, right column, bottom paragraph; (ii) the production of transgenic rats that expressed an additional rat *Ren-2* gene and that exhibited hypertension and associated phenotypes, *see* Lee, M.A. *et al., Am. J. Physiol. 270*:E919-E929 (1996), *see* especially page E921, left column, first full paragraph; (iii) the production of transgenic mice and a transgenic pig that expressed the human complement inhibitor hCD59, the cells of which exhibited resistance to challenge with high-titer anti-porcine antibody and human complement, *see* Fodor, W.L. *et al., Proc. Natl. Acad. Sci. USA 91*:11153-11157 (1994), *see* especially page 11157, left column, last full paragraph; and (iv) the production of a transgenic pig that expressed a murine leukemia virus-rat somatotropin fusion gene and that produced high levels of circulating rat somatotropin and exhibited increased skeletal growth and reduced fat deposition, *see* Ebert, K.M. *et al., Mol. Endocrinol. 2*:277-283 (1988), *see* especially paragraph bridging pages 280-281. These references demonstrate that producing transgenic animals having particular phenotypes would not have required undue experimentation.

In view of the teachings of the specification and the knowledge possessed by those of ordinary skill in the art, it would have required only routine experimentation to create and use the transgenic animals encompassed by claims 7-9, 35 and 36.

(b) Claims Directed to Methods for Screening Candidate Drugs are Fully Enabled

Claims 14-16 and new claims 37 and 38 are directed to *in vivo* methods for screening a candidate drug that is potentially useful for the treatment or prevention of Alzheimer's disease, neuroectodermal tumors, malignant astrocytomas, and glioblastomas. The methods of current claims 14-16 comprise: (a) administering a candidate drug to the transgenic animal of claim 7, and (b) detecting at least one of the following: (i) the suppression or prevention of expression of the protein coded for by the DNA molecule contained by said animal; or (ii) the increased degradation of the protein coded for by the DNA construct contained by said animal; due to the drug candidate compared to a control animal which has not received the candidate drug. The methods of new claims 37 and 38 comprise: (a) administering a candidate drug to the transgenic animal of claim 7, wherein said transgenic animal exhibits at least one of neuritic sprouting, nerve cell death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons; and (b) detecting the reduction of frequency of at least one of neuritic sprouting, nerve cell death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons in the host due to the drug candidate compared to a control animal which has not received the candidate drug.

A person of ordinary skill in the art would have been able to practice the methods encompassed by the claims using routine methods in the art. The methods of the invention involve the use of the transgenic animal encompassed by claim 7. As demonstrated above, it would have required only routine experimentation for a skilled artisan to obtain transgenic animals encompassed by claim 7, including transgenic animals of claim 7 that exhibit at least one of neuritic sprouting, nerve cell death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons. Moreover, the administration of a candidate drug to the transgenic animal of claim 7 would have been a matter of routine procedure in the art.

In addition, a person of ordinary skill in the art could have performed the detecting aspects of current claims 14-16 with only routine experimentation. Specifically, detecting: (i) the suppression or prevention of expression of the protein coded for by the DNA molecule of SEQ ID NO:1 or a DNA molecule which is at least 90% homologous thereto, and (ii) the increased degradation of the protein, would have been a matter of routine procedure in the art. As discussed in the specification, the suppression or prevention of expression, and the increased degradation of the protein, could have been detected, for example, with antibodies specific for AD7c-NTP. *See* specification at page 21, lines 21-22. As noted in the specification:

> Monoclonal and polyclonal antibodies which are specific for AD7c-NTP as well as methods for the qualitative and quantitative detection of AD7c-NTP are described herein as well as in WO94/23756 and U.S. appl. no. 08/340,426. Such testing may be carried out on CSF of the transgenic animal or by immunohistochemical staining of a tissue section from the brain of the animal. In addition, such testing may be carried out by Western blot analysis, ELISA or RIA.

Specification at page 21, lines 23-29. Other methods for monitoring protein expression, such as RT-PCR and *in situ* hybridization, would have been available to one of ordinary skill in the art as well. *See* specification at page 42, line 1 through page 43, line 24.

A person of ordinary skill in the art could also have performed the detecting aspects of new claims 37 and 38 with only routine experimentation. That is, detecting the reduction of frequency of at least one of neuritic sprouting, nerve cell death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons in the host would have required only routine experimentation in the art. As noted in the specification, the frequency of these phenotypes could have been determined easily using, *e.g.*, immunohistochemical staining. *See* specification at page 22, lines 1-12. The *in vitro* results presented in the specification relating to AD7c-NTP over-expression would have provided additional guidance for one skilled in the art to detect the reduction of frequency of at least one of the recited cellular phenotypes. *See, e.g.*, specification at page 46, lines 1-26 and Figs. 5 and 6A-6G.

In view of the teachings of the specification and the knowledge possessed by those of ordinary skill in the art, it would have required only routine experimentation to practice the full scope of the methods encompassed by claims 14-16 and new claims 37 and 38.

2. A Prima Facie Case of Non-Enablement Has Not Been Established

In order to establish a *prima facie* case of lack of enablement, the Examiner has the initial burden to set forth a reasonable basis to question the enablement provided for the claimed invention. *See In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993). To satisfy this burden, "it is incumbent upon the Patent Office... to explain *why*

it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement." *See In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971) (emphasis in original). As discussed in detail below, the Examiner has not presented any specific evidence or sound scientific reasoning to indicate that the present claims are not enabled. Thus, a *prima facie* case of non-enablement has not been established.

The Examiner first stated that "the claimed invention is not supported by a sufficient written description." Paper No. 14, page 9. As discussed above, the specification provides adequate written description for the subject matter encompassed by Applicants' currently presented claims. A person of ordinary skill in the art, based on the specification, would appreciate that Applicants' were in possession of the claimed subject matter. Therefore, the rejection for non-enablement cannot properly be based on the supposed insufficiency of the description of the invention.

The Examiner next stated that "the as-filed specification does not provide sufficient guidance for one skilled in the art to make and/or use any DNA molecule which is at least 90% homologous to SEQ ID NO: 1." Paper No. 14, page 9. The basis for this assertion is the supposed "unpredictability of the relationship between sequences and function." *See* Paper No. 14, page 10. The Examiner, however, has not explained why obtaining DNA molecules for use with the present invention would require one skilled in the art to predict protein function from sequence information.

As discussed in Applicants' previous response, a skilled artisan would not need to be able to predict the structural and/or functional consequences of particular mutations or base changes in order to produce DNA molecules that are 90% homologous to SEQ ID

NO:1 and that code for proteins having an activity of AD7c-NTP when over-expressed in neuronal cells. *See* the August 8, 2002 Response at pages 17-19. To make the DNA molecules that are included within the transgenic animals of the present invention, the skilled artisan would have only needed to: (a) obtain DNA molecules that are at least 90% homologous to SEQ ID NO:1, and (b) test them for the ability to encode proteins that possess AD7c-NTP activity when over-expressed in neuronal cells. As discussed in Applicants' previous response, and in *Section II.B(1)(a)(i)* above, both of these processes would have been routine in the art.

Since a skilled artisan would not have been required to predict the consequences of sequence changes on protein structure and/or function in order to obtain DNA molecules for use with the present invention, the enablement rejection cannot properly be based on this asserted unpredictability.

Applicants note that the rejection for lack of enablement appears to be based, at least in part, on the phrase "wherein said DNA molecule codes for a protein that has an activity of AD7c-NTP when *expressed* in neuronal cells," recited in claim 7. *See, e.g.*, Paper No. 14, page 20. Applicants note that claim 7 has been amended to recite "wherein said DNA molecule codes for a protein that has an activity of AD7c-NTP when *over*-expressed in neuronal cells." (Emphasis added). The purpose of this amendment is to make explicit an implicit feature of the invention. Thus, the rejection, insofar as it relates to the recitation of the phrase "wherein said DNA molecule codes for a protein that has an activity of AD7c-NTP when *expressed* in neuronal cells," has been fully accommodated.

The Examiner next stated that "the as-filed specification does not provide sufficient guidance or factual evidence for any transgenic animal expressing a nucleotide sequence

encoding SEQ ID NO: 1 or a DNA molecule, which is at least 90% homologous thereto, and any corresponding phenotype." Paper No. 14, page 11. To support this conclusion the Examiner asserted that "the as-filed specification only contemplates the use of embryonic stem (ES) cell technology or using pro-nuclear injection for the generation of transgenic mammals for [use] in the claimed invention." *See* Paper No. 14, page 11. As noted in Applicants' previous reply, this is an incorrect statement. *See* the August 8, 2002 Response at pages 21-22. First, embryonic stem cell technology and pronuclear microinjection are only two exemplary methods that can be used to produce the transgenic animals of the present invention. Second, the specification indicates that any method known to those skilled in the art can be used for the production of the transgenic animals of the invention. *See* specification at page 20, lines 18-19 and Lazzarini at column 13, lines 2-12 (which is cited and incorporated by reference in the specification). Third, an applicant is not limited to the confines of the specification to provide the necessary information to enable an invention. *See Howarth*, 654 F.2d at 105-6, 210 USPQ at 692. Thus, in order to generate the transgenic animals of the present invention, a person of ordinary skill in the art would have had at his or her disposal any method of transgenic animal production that was known in the art at the time of the application.

In addition, the enablement requirement of 35 U.S.C. § 112, first paragraph, is satisfied as long as *at least one* method of making the claimed subject matter is enabled. *See Johns Hopkins Univ. v. CellPro, Inc.*, 152 F.3d 1342, 1361, 47 USPQ2d 1705, 1719 (Fed. Cir. 1998) ("the enablement requirement is met if the description enables any mode of making and using the invention.") The Examiner therefore can only satisfy his burden under 35 U.S.C. § 112, first paragraph, by showing that, at the time the application was filed, *all*

known methods for producing transgenic animals required the expenditure of undue experimentation. Since no such evidence or assertions have been put forth, a *prima facie* case of non-enablement has not been established.

Despite the fact that multiple methods for producing transgenic animals were available to those skilled in the art at the time the application was filed, the Examiner, in explaining the enablement rejection, has focused his attention on only two exemplary methods: pronuclear microinjection and embryonic stem cell technology. This analysis is incomplete since many other methods were available to the skilled artisan. The Examiner has not set forth any evidence or sound scientific reasoning that would indicate that *other methods* for producing transgenic animals would require undue experimentation. Moreover, the Examiner has not provided evidence that would indicate that transgenic animal production using pronuclear microinjection or embryonic stem cell technology would entail a degree of experimentation that would be regarded as undue in the context of the present invention. *See* the August 8, 2002 Response at pages 22-26.

The Examiner has cited various references that supposedly support the assertion that it would require undue experimentation to produce the transgenic animals of the invention. As discussed below, none of the references cited by the Examiner, alone or in combination, are sufficient to establish a *prima facie* case of non-enablement. In certain instances, the references cited by the Examiner actually indicate that producing transgenic animals would have been routine in the art.

The Examiner first cited Polejaeva *et al.*, *Theriogenology 53*:117-126 (2000). As noted in Applicants' previous response, Polejaeva explicitly states that "[t]ransgenic animals *can be successfully produced in a number of species* including mice, rabbits, pigs, sheep,

cattle and goats by the injection of the gene of interest into the pronucleus of a zygote." *See* Polejaeva at page 119, second full paragraph (emphasis added, internal citations omitted). Thus, Polejaeva indicates that transgenic animal production using pronuclear microinjection would *not* entail undue experimentation.

Polejaeva goes on to describe the "limitations" that are associated with pronuclear injection. The "most profound limitation," mentioned by Polejaeva, is that DNA can only be added, not deleted or modified *in situ*. *See id.* Since the production of transgenic animals that are included within Applicants' claims only requires the addition of DNA molecules, this "most profound limitation" is inapplicable to Applicants' invention.

The other two limitations of pronuclear microinjection cited by Polejaeva are (a) the potential for random integration of foreign DNA, and (b) the possibility of creating mosaic animals. *See id.* Aside from citing Polejaeva for the proposition that random integration and the production of mosaics may be a "limitation" in the practice of pronuclear microinjection to produce transgenic animals, the Examiner has not set forth any evidence that would suggest that this method would require an "undue amount of experimentation." The potential limitations associated with pronuclear microinjection only indicate that a certain level of experimentation may be needed to create transgenic animals using this technology. The necessity of some experimentation to practice a claimed invention does not render an invention non-enabled as long as the quantity of experimentation needed is not regarded as undue. *See In re Angstadt*, 537 F.2d 498, 504, 190 USPQ 214, 219 (CCPA 1976).

There is no indication that the experimentation needed to successfully obtain transgenic animals using pronuclear microinjection would be regarded as *undue*. Indeed,

the fact that transgenic animals can be, and have been, successfully produced using pronuclear microinjection indicates that the practice of this technology is not regarded as requiring undue experimentation. *See* Polejaeva at page 119, second full paragraph. In addition, others in the field have noted that:

> Pronuclear DNA injection has enabled the scientific community world wide to selectively add defined genes of choice into the germ line of laboratory as well as farm animals. Many experiments with transgenic animals confirmed that transgenesis can provide new insight into many aspects of mammalian life, development and diseases.

See Rülicke and Hübscher, *Exp. Physiol. 85*:589-601 (2000) at page 597, left column, first full paragraph.

In addition, the Examiner has misquoted an important sentence from Polejaeva[1]. The Examiner quotes Polejaeva as stating: "Therefore, the production of the required phenotype coupled to germ line transmission could [require (?)] undue experimentation." *See* Paper No. 14, page 12. The actual quote is as follows: "Therefore, the production of the required phenotype coupled to germ line transmission could require the generation of several transgenic founder lines." *See* Polejaeva at page 119, second full paragraph. Far from indicating that the use of pronuclear injection involves "undue experimentation," Polejaeva merely notes that the "limitations" associated with this methodology may require that several transgenic founder lines be produced. There is nothing to suggest that producing multiple founder lines is of such difficulty as to satisfy the legal standard of "undue experimentation."

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[1]Applicants pointed out this error in their previous response. *See* the August 8, 2002 Response at page 25. Nevertheless, in the present Office Action, the Examiner has repeated the misquotation of Polejaeva.

Moreover, the final two sentences in the cited paragraph of Polejaeva indicate that alternative methods are available for producing transgenic animals that may avoid the limitations of pronuclear injection:

> Somatic cell nuclear transfer [described on page 120 of Polejaeva] will eliminate this problem and accelerate transgenic herd or flock generation. In addition, transgenic sheep produced using this new technology require the use of fewer than half the animals needed for pronuclear microinjection.

See Polejaeva at page 119, second full paragraph. Therefore, Polejaeva indicates that somatic cell nuclear transfer (a method that would have been available to one of ordinary skill in the art at the time of the application) is another method that is likely to be successful in the production of transgenic animals.

The Examiner next turned his attention to ES cell technology. The Examiner asserted that ES cell technology "is generally limited to the mouse system." *See* Paper No. 14, page 12. To support this position, the Examiner cited Rülicke and Hübscher, *Exp. Physiol. 85*:589-601 (2000) and Bishop, J.O., *Reprod. Nutr. Dev. 36*:607-618 (1996). Applicants emphasize that many other methods for producing non-mouse transgenic animals were available to those skilled in the art as of the effective filing date of the application. *See, e.g.*, U.S. Patent No. 5,602,299 (Lazzarini), column 12, line 65, through column 13, line 15. Applicants also emphasize that the invention is sufficiently enabled as long as at least one of the known methods of transgenic animal production could have been used to make the transgenic animals of the present invention. *See Johns Hopkins Univ.,* 152 F.3d at 1361, 47 USPQ2d at 1719. Therefore, the contention that ES cell technology "is generally limited

to the mouse system" does not provide sufficient evidence to indicate that making and using the transgenic animals of the invention would have required undue experimentation.

The Examiner next cited Trojanowski and Lee, *Brain Pathology 9*:733-739 (1999) (hereinafter "Trojanowski") for the proposition that "certain characteristic[s] can be produced in a test tube, [but] the conditions required are highly artificial and in vitro paradigms have limited utility as models of in vivo mechanisms of neurodegeneration." Paper No. 14, page 13. Trojanowski, however, simply emphasizes the need in the art for the development of additional transgenic mouse models of filamentous brain lesions. *See* Trojanowski at page 733. Trojanowski does not indicate or suggest that the production of transgenic animals would have required undue experimentation. In fact, Trojanowski reports the successful production of transgenic mouse lines that over-expressed the tau protein and that exhibited "pre-tangle" tau pathology. *See* Trojanowski at page 736, left column. Therefore, Trojanowski does not support the position that the claimed invention is not enabled.

The Examiner then focused on the alleged failure of the specification to describe any particular phenotype exhibited by the transgenic animals of the invention. Specifically, the Examiner stated:

> [T]he disclosure fails to provide any relevant teachings or sufficient guidance with regards to the production of any transgenic animals comprising a transgenic sequence encoding SEQ ID NO: 1 or a sequence with 90% homology thereto, which expresses the transgenic sequence such that a phenotype occurs. Furthermore, the as-filed specification fails to describe any particular phenotype exhibited by any contemplated transgenic animal of the invention when the nucleotide sequence is expressed and not over-expressed in said animal.

Paper No. 14, page 14. Applicants respectfully disagree with these statements.

The Examiner acknowledged that "the claimed transgenic mammal [sic: animal] is not limited to the expression of the protein at a level resulting in a specific phenotype," but that the broadest interpretation of the claims "consistent with the specification" is such that they encompass a:

> transgenic mammal [sic: animal] having cells, which harbor a recombinant nucleic acid that expresses the protein at a level sufficient to result in a specific phenotype (i.e., it is unknown what other purpose the transgenic mammal [sic: animal] would serve if the transgene (e.g. SEQ ID NO: 1 or a sequence with 90% homology thereto) is not expressed at a sufficient level for a resulting phenotype).

See Paper No. 14, page 14. Applicants respectfully disagree. None of the currently presented claims, aside from new claims 37-39, can be interpreted as requiring that the transgenic animals exhibit any particular phenotype.

The Examiner stated that "the teachings of the specification are to be taken into account because the claims are to be given their broadest reasonable interpretation that is consistent with the specification." *See* Paper No. 14, page 14. The Examiner, however, has not pointed to anything in the specification that would support his claim interpretation. That is, nothing in the specification is cited to support the interpretation of claims 7-9, 14-16, 35 and 36 as requiring that the transgenic animals exhibit a specified phenotype.

As noted in Applicants' previous response, claims 7-9, 14-16, 35 and 36 do not require that the transgenic animals exhibit any particular phenotype. *See* the August 8, 2002 Response at pages 28-30. There is nothing in the specification that would indicate or suggest that the transgenic animals encompassed by or included within these claims must exhibit any particular phenotype. Transgenic animals encompassed by or included within

the subject matter of claims 7-9, 14-16, 35 and 36 are useful in, *e.g.*, drug screening applications even if they do not exhibit any specific phenotypes.

One manner by which drugs useful in the treatment or prevention of Alzheimer's disease can be identified is by administering candidate drugs to a transgenic animal whose germ and somatic cells comprise the DNA molecule of SEQ ID NO:1 or a DNA molecule which is at least 90% homologous thereto, and identifying those drugs that cause, *e.g.*, the suppression or prevention of expression of the protein encoded by the DNA molecule contained by the transgenic animal. *See* specification at page 21, line 12. Alternatively, drugs can be identified on the basis of their ability to increase the degradation of the protein encoded by the DNA molecule contained by the transgenic animal. *See* specification at page 21, line 13. Therefore, the only characteristic that the transgenic animals encompassed by or included within the subject matter of claims 7-9, 14-16, 35 and 36 need to possess in order to be useful for the contemplated screening methods is that they express the DNA molecule of SEQ ID NO:1 or a DNA molecule that is at least 90% homologous thereto.

New claims 37-39 specify that the transgenic animal used in the claimed methods exhibits at least one of neuritic sprouting, nerve cell death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons. The Examiner has cited four references that supposedly illustrate the difficulties associated with producing transgenic animals that exhibit a particular phenotype: Wall, R.J., *Theriogenology 45*:57-68 (1996) (hereinafter "Wall"), Houdebine, L-M., *J. Biotechnology 34*:269-287 (1994), Mullins, L.J. and Mullins, J.J., *J. Clin. Invest. 97*:1557-1560 (1996) (hereinafter "Mullins"), and Strojek, R.M. and Wagner, T.E., *Genetic Engineering: Principles and Methods 10*:221-246 (1988). *See* Paper No. 14, page 15. The cited references, however, do not support a *prima facie* case

of non-enablement. The references simply point out various technical considerations that a skilled artisan might need to address in producing transgenic animals. *See* the August 8, 2002 Response at pages 29-30. The need for experimentation does not render an invention non-enabled unless the quantity of experimentation needed is regarded as undue. *See Angstadt*, 537 F.2d at 504, 190 USPQ at 219. The references cited by the Examiner do not suggest that the amount of experimentation needed to produce transgenic animals with a particular phenotype would be considered undue. Some of the references cited by the Examiner actually suggest that producing transgenic animals with particular phenotypes was routine in the art.

There are numerous examples in the art -- *including the references cited by the Examiner* -- that describe the successful production of transgenic animals possessing specific phenotypes. Wall, for example, summarizes the results of various researchers demonstrating the production of: (i) transgenic sheep with enhanced wool production characteristics, *see* Wall at page 59, third full paragraph; (ii) transgenic mice that serve as models for human genetic diseases (including Alzheimer's disease), *see id.*; and (iii) transgenic pigs that express a human complement inhibitor for use in xenograft transplantation. *See id.* Mullins describes examples of the production of: (i) transgenic rats that successfully express the human apolipoprotein A-1 gene and that show increased serum HDL cholesterol concentrations, *see* Mullins at page 1558, right column, first full paragraph; (ii) transgenic rabbits expressing apoB-editing protein in the liver and exhibiting reduced LDL and lipoprotein(a) concentrations, *see id.*; (iii) transgenic rabbits expressing the human CD4 protein on T lymphocytes and exhibiting susceptibility to HIV infection, *see id.*; (iv) transgenic pigs expressing a bovine growth hormone gene and the resultant consequences

on carcass tissue lipid composition, *see id.* at page 1558, paragraph bridging pages 1558-1559 (reference 23); and (v) transgenic swine expressing high levels of human hemoglobin, *see id.* Moreover, Applicants, in their previous response, have submitted multiple references showing the successful production of transgenic animals expressing specific phenotypes. *See Section III.B(1)(a)(ii)* above, and the August 8, 2002 Response at pages 31-32.

The fact that there are numerous examples in the art of successfully produced transgenic animals expressing specific desired phenotypes indicates that the production of such transgenic animals would not have required undue experimentation.

The Examiner also made certain comments that are directed to the enablement of method claims 14-16. *See* Paper No. 14, pages 16-17. The Examiner, however, has not presented specific evidence or sound scientific reasoning to indicate that the methods of the invention are not enabled. The Examiner stated that "[t]he art of record teaches that there is no animal model that can mimic all the cognitive, behavorial. [sic: behavioral,] Biochemical, and histopathological abnormalities observed in a patient with AD (Yamada et al., Pharmacology & Therapeutic, Vol. 88, 93-113, 2000)." Paper No. 14, page 17. The Examiner has not indicated why the supposed absence of such an animal model would render Applicants' claimed methods non-enabled. As discussed above, the methods of present claims 14-16 comprise detecting at least one of the following: (i) the suppression or prevention of expression of the protein coded for by the DNA construct contained by the transgenic animal of claim 7; or (ii) the increased degradation of the protein coded for by the DNA construct contained by the animal. New claims 37 and 38 comprise detecting the reduction of frequency of at least one of neuritic sprouting, nerve cell death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons in the host.

The methods of claims 14-16, 37 and 38, would *not* require that the transgenic animal exhibit all the cognitive, behavioral, biochemical and histopathological abnormalities associated with Alzheimer's disease. All that is required is that the DNA molecule be expressed in the cells of the transgenic animal (claims 14-16), or that the transgenic animal exhibit at least one of neuritic sprouting, nerve cell death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons (claims 37 and 38). Thus, whether or not a model organism has been described in the art that exhibits all the cognitive, behavioral, biochemical and histopathological abnormalities associated with Alzheimer's disease does not have any bearing on the enablement of the claimed methods.

The Examiner also stated that "the art of record is absent about a drug that is able to treat or prevent any of these diseases listed above. . ." Paper No. 14, page 17. The absence in the art of a particular teaching, however, cannot satisfy the Examiner's burden of presenting specific evidence to indicate that the invention is not enabled. *See Gould v. Quigg*, 822 F.2d 1074, 1078, 3 USPQ2d 1302, 1304 (Fed. Cir. 1987) ("The mere fact that something has not previously been done clearly is not, in itself, a sufficient basis for rejecting all applications purporting to disclose how to do it;" quoting *In re Chilowsky*, 229 F.2d 457, 461, 108 USPQ 321, 325 (CCPA 1956)).

The Examiner also stated:

> Furthermore, it is not apparent to one skilled in the art how to perform an assay for screening a candidate drug of [sic: for] Alzheimer's disease, neuroectodermal tumors, malignant astrocytomas, and glioblastomas in a transgenic animal comprising a nucleotide sequence that is 90% homologous to SEQ ID NO: 1 if a phenotype comprising neuritic sprouting, nerve cell death, nerve cell degeneration, neurofibrillary tangles, and/or irregular swollen neurites in neuronal cells is not observed in the transgenic animal.

Paper No. 14, page 23.

Applicants note that, of the currently presented claims, only new claims 37 and 38 comprise detecting the reduction of frequency of at least one of neuritic sprouting, nerve cell death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons in the host. Applicants further note that claims 37 and 38 specify that the transgenic animal to which a candidate drug is administered exhibits at least one of neuritic sprouting, nerve cell death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons. Thus, the Examiner's comments, quoted above, are rendered moot in view of the amendment to claim 14, and cannot apply to new claims 37 and 38.

3. Summary

A person of ordinary skill in the art, in view of the specification, would have been able to make and use the transgenic animals of claims 7-9, 35 and 36 using only routine methods. In addition, a person of ordinary skill in the art, in view of the specification, would have been able to practice the full scope of the methods encompassed by claims 14-16 (and of new claims 37 and 38) with only routine experimentation. The Examiner has not presented any specific evidence or sound scientific reasoning that would indicate that making the claimed transgenic animals, or practicing the claimed methods would have required undue experimentation. Thus, a *prima facie* case of non-enablement has not been established. In view of the foregoing, Applicants respectfully request that the rejection of claims 7-9, 14-16, 35 and 36 under 35 U.S.C. § 112, first paragraph, for lack of enablement, be reconsidered and withdrawn.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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Version with markings to show changes made

Please substitute the following claim 7 for the pending claim 7:

7. (Twice amended) A transgenic non-human animal, all of whose germ and somatic cells comprise the DNA molecule of SEQ ID NO:1 or a DNA molecule which is at least 90% homologous thereto, wherein said DNA molecule is over-expressed in one or more cells of said transgenic animal, and wherein said DNA molecule codes for a protein that has an activity of AD7c-NTP when over-expressed in neuronal cells.

Please substitute the following claim 14 for the pending claim 14:

14. (Once amended) An *in vivo* method for screening a candidate drug that is potentially useful for the treatment or prevention of Alzheimer's disease, neuroectodermal tumors, malignant astrocytomas, and glioblastomas, [which comprises] said method comprising:

(a) administering a candidate drug to the transgenic animal of claim 7, and

(b) detecting at least one of the following:

(i) the suppression or prevention of expression of the protein coded for by the DNA molecule [construct] contained by said animal; or

(ii) the increased degradation of the protein coded for by the DNA construct contained by said animal[; or

(iii) the reduction of frequency of at least one of neuritic sprouting, nerve cell death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons in the host;]

due to the drug candidate compared to a control animal which has not received the candidate drug.

Please substitute the following claim 36 for the pending claim 36:

36. (Once amended) The transgenic non-human animal of claim 7, wherein said activity of AD7c-NTP possessed by said DNA molecule when <u>over-</u>expressed in neuronal cells is selected from the group consisting of neuritic sprouting, nerve cell death, nerve cell degeneration, neurofibrillary tangles and irregular swollen neurites.

Please add new claims 37-40.

EXHIBIT 1

At present, it is impossible to predict with accuracy the effect of substituting one amino acid for another in a protein. Current attempts to "improve" the properties of a protein therefore depend on analyzing large numbers of variants that are created by site-directed mutagenesis in promising regions (e.g., in and around the active site of an enzyme). Clearly, the number of potential variations that can be created, even in a circumscribed region of a protein, is extremely large. For example, 114 different mutants would be required simply to insert every possible amino acid at each of six locations in a protein. This number grows to 6^{19} if such substitutions are made in a combinatorial fashion. When planning this type of mutagenesis, careful choices must therefore be made to keep the numbers of mutants within manageable limits. For example, the numbers of potential mutants can be markedly reduced by avoiding replacements that are (1) highly conservative (i.e., the substitution of one amino acid with another whose chemical properties are very similar), (2) highly radical (i.e., replacing an amino acid with another whose chemical properties are completely different), or (3) misguided (e.g., the substitution of cysteine residues in secretory proteins). However, when the number of desired mutants exceeds 20 or so, it becomes impractical and expensive to generate each of them individually using a separate mutagenic oligonucleotide. Methods have therefore been devised to use degenerate pools of oligonucleotides to create large populations of mutants in a single round of site-directed mutagenesis. These degenerate pools of oligonucleotides contain a mixture of normal and abnormal bases at each position in the sequence at which a mutagenic event is desired. In the remainder of this section, we present guidelines for ways in which these populations of clustered mutations can be efficiently generated using degenerate pools of mutagenic oligonucleotides.

Use of Degenerate Pools of Mutagenic Oligonucleotides

1. Pools of degenerate single-stranded oligonucleotides can be used only when the target amino acids are clustered. If all of the codons that are to be altered lie within a short stretch of contiguous nucleotides, a pool of degenerate single-stranded mutagenic oligonucleotides can be used as mismatched primers on single-stranded DNA templates to generate the corresponding set of mutants. However, the mutants cannot generally be distinguished from the original wild-type DNA by the standard method of screening by hybridization. In most cases, the mutagenic oligonucleotides are so long that there is no practical difference in stability between mismatched and perfect hybrids. Even if the oligonucleotides are sufficiently short (≤ 20 nucleotides in length), the pool usually contains many different members, each of which has different hybridization characteristics. It is therefore extremely difficult, if not impossible, to devise hybridization conditions that will distinguish all possible mutant sequences from the original wild-type sequences. This type of mutagenesis is therefore best carried out using the Kunkel system (see pages 15.74–15.79), which selects strongly against bacteriophages generated by replication of the original wild-type (+) strand of DNA. Mutants are then identified by picking individual plaques blindly and sequencing the relevant section of single-stranded bacteriophage DNA.

2. An alternative method is to generate pools of mutants by "cassette mutagenesis," a technique that involves replacing the wild-type sequence with synthetic double-stranded oligonucleotides (see, e.g., McNeil and Smith 1985; Wells et al. 1985; Derbyshire et al. 1986; Hill et al. 1986, 1987; Hutchison et al. 1986; Bedwell et al. 1989). Since cassette mutagenesis was first introduced (Matteucci and Heyneker 1983), several variations have been described, each of which has advantages under particular circumstances. However, all of these techniques suffer from a common drawback—the necessity for unique restriction sites at both ends of the cassette. Because these restriction sites are required to shuttle the synthetic double-stranded oligonucleotide into the correct location, they cannot occur anywhere else in either the plasmid vector or the segment of the wild-type gene that it carries. Furthermore, to ensure that the cassette is inserted in the correct orientation, the cassette should carry different restriction sites at each end. Because naturally occurring restriction sites hardly ever fulfill these criteria, it is usually necessary to carry out one or more rounds of site-directed mutagenesis to create suitable restriction sites at the appropriate locations in the wild-type gene. If the introduction of these sites changes the amino acid sequence encoded by the gene, it is necessary to determine whether the resulting protein displays wild-type characteristics. To eliminate the possibility that the phenotypes of any mutants obtained by cassette mutagenesis result from a combination of amino acid changes (i.e., changes caused by introduction of the restriction sites and by changes encoded within the cassette), it may be necessary to restore the original wild-type sequence at the restriction sites.

   Three different methods are currently used to generate double-stranded

oligonucleotide cassettes. In the first method (McNeil and Smith 1985) (see Figure 15.9A), two separate sets of oligonucleotides are synthesized that are complementary to the opposite strands of the target DNA. One of these sets consists of a single species of oligonucleotide that is exactly complementary to the sequence of one of the strands of the wild-type target DNA. The other set consists of a degenerate pool of oligonucleotides that is complementary to the opposite strand and that carries the desired mutations. These sets of complementary oligonucleotides are then mixed under conditions that will allow mismatched hybrids to form. If the complementary oligonucleotides have been designed to yield double-stranded cassettes that carry the appropriate protruding termini, they can be inserted directly into a recombinant plasmid in place of the homologous wild-type sequence. Alternatively, cohesive termini can be created by digesting double-stranded blunt-ended cassettes with the appropriate restriction enzymes. The mismatches in the recombinant plasmids are repaired in vivo, after the recombinant plasmids have been introduced into competent bacteria. Subsequent replication of the plasmid DNA and segregation into daughter cells allows clones to be isolated that are derived from each DNA strand of the plasmid originally used for transformation. In this method and the one that follows, the plasmids isolated from individual colonies of transformed bacteria are occasionally heterogeneous, suggesting that segregation of the plasmids is sometimes incomplete. This problem can be solved by retransforming competent bacteria with plasmid DNAs extracted from pooled primary transformants. However, in this first method of cassette mutagenesis, the frequency of mutation can never exceed 50% because only one half of the progeny plasmids are derived from the mutagenized strand.

In the second method (see Figure 15.9B), the frequency of mutation is increased by using cassettes in which the complementary strands both consist of mixed-sequence oligonucleotides (Wells et al. 1985). Because each of these strands gives rise to progeny plasmids, the mutation rate can be raised to greater than 50% (Makris et al. 1988).

In the third method (see Figure 15.9C), degenerate pools of single-stranded oligonucleotides are converted to a blunt-ended double-stranded form by mutually primed synthesis (Oliphant et al. 1986; Hill et al. 1987). Two degenerate pools of oligonucleotides are synthesized that are complementary to the same strand of the target DNA. However, the members of one pool carry sequences at their 3′ termini that are complementary to sequences at the 3′ termini of oligonucleotides in the second pool. Usually, these complementary sequences are palindromic and correspond to the restriction site that marks one end of the cassette. The oligonucleotides in the two pools are then annealed to form partial hybrids that can be converted to blunt-ended double-stranded DNA by the Klenow fragment of *E. coli* DNA polymerase I. The products of this reaction are tail-to-tail dimers. Unit-length cassettes are generated by digesting the dimers with the appropriate restriction enzymes.

The major advantage of the third method is that the unit-length cassette consists of perfect homoduplexes. Any potential bias that occurs during mismatch repair in vivo is therefore avoided, and there is no loss of

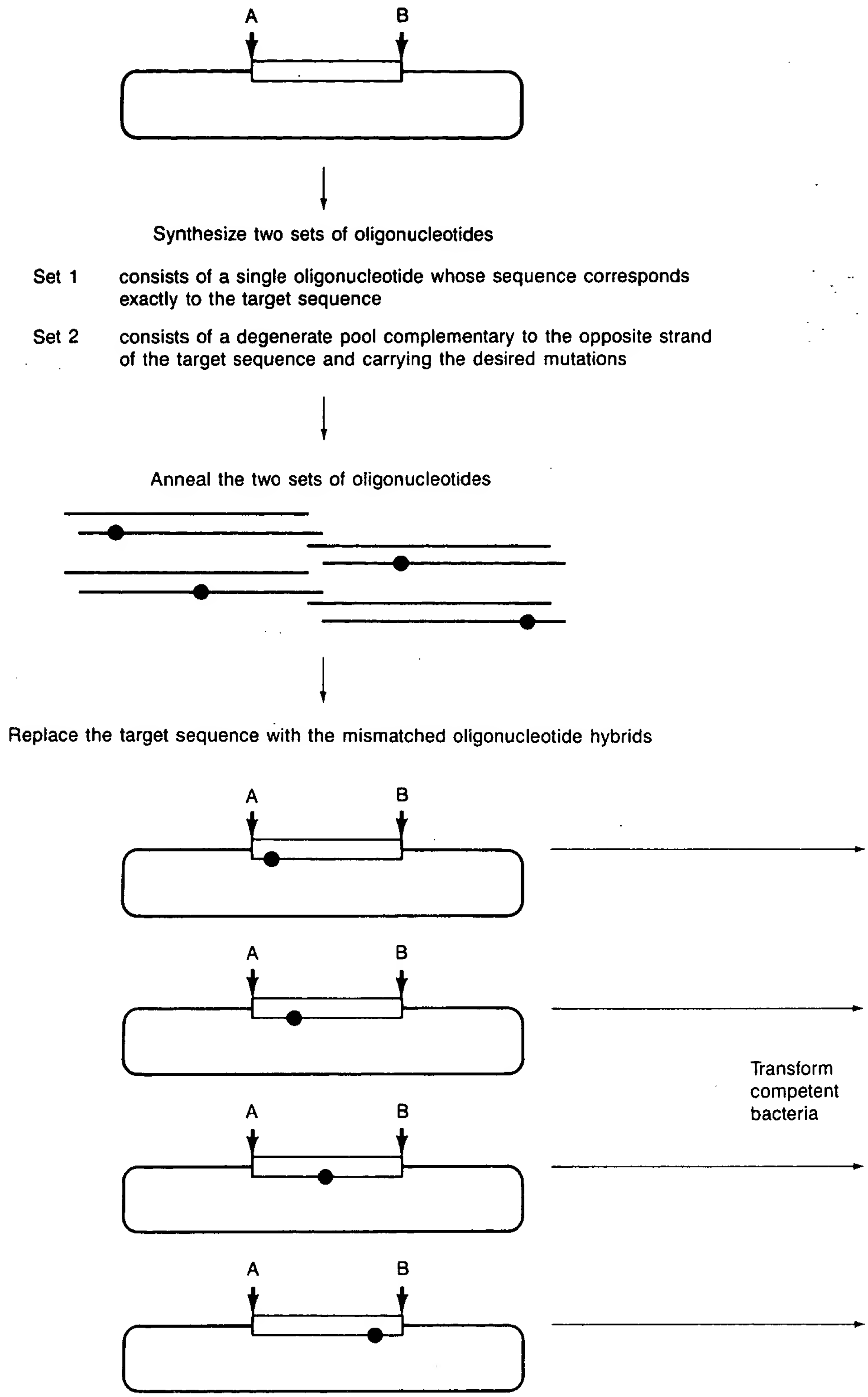
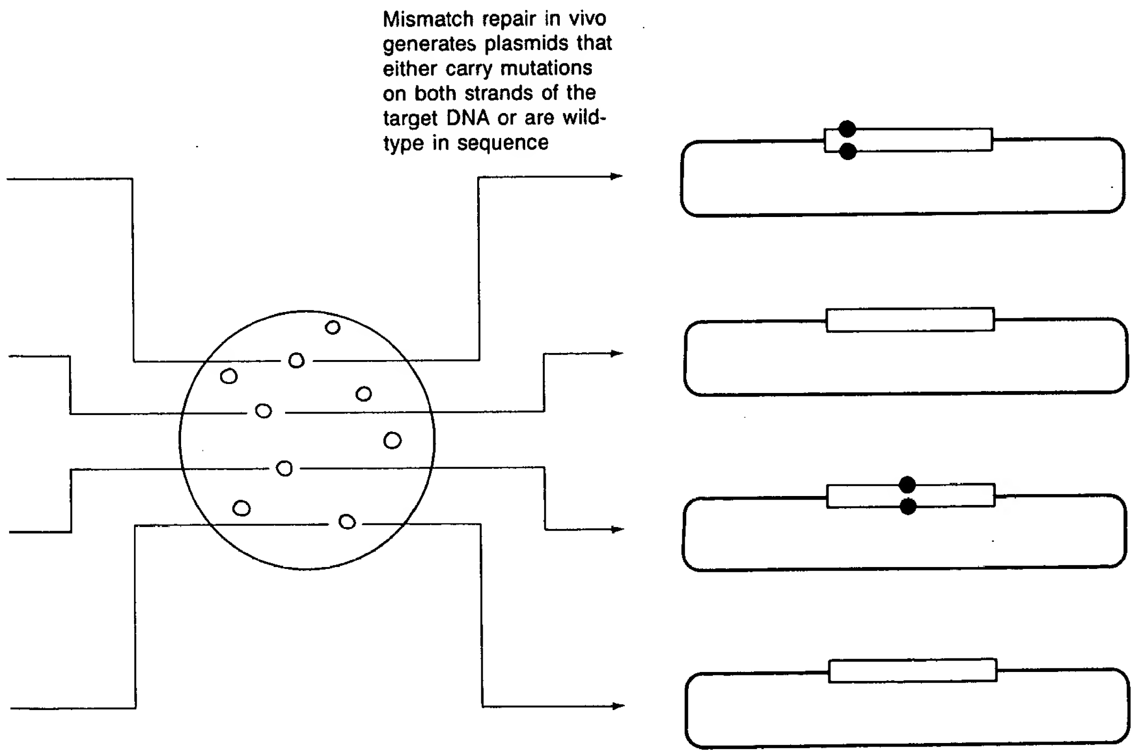


FIGURE 15.9A
Cassette mutagenesis using a single mixed-sequence oligonucleotide and repair of mismatches in vivo.


Mismatch repair in vivo generates plasmids that either carry mutations on both strands of the target DNA or are wild-type in sequence

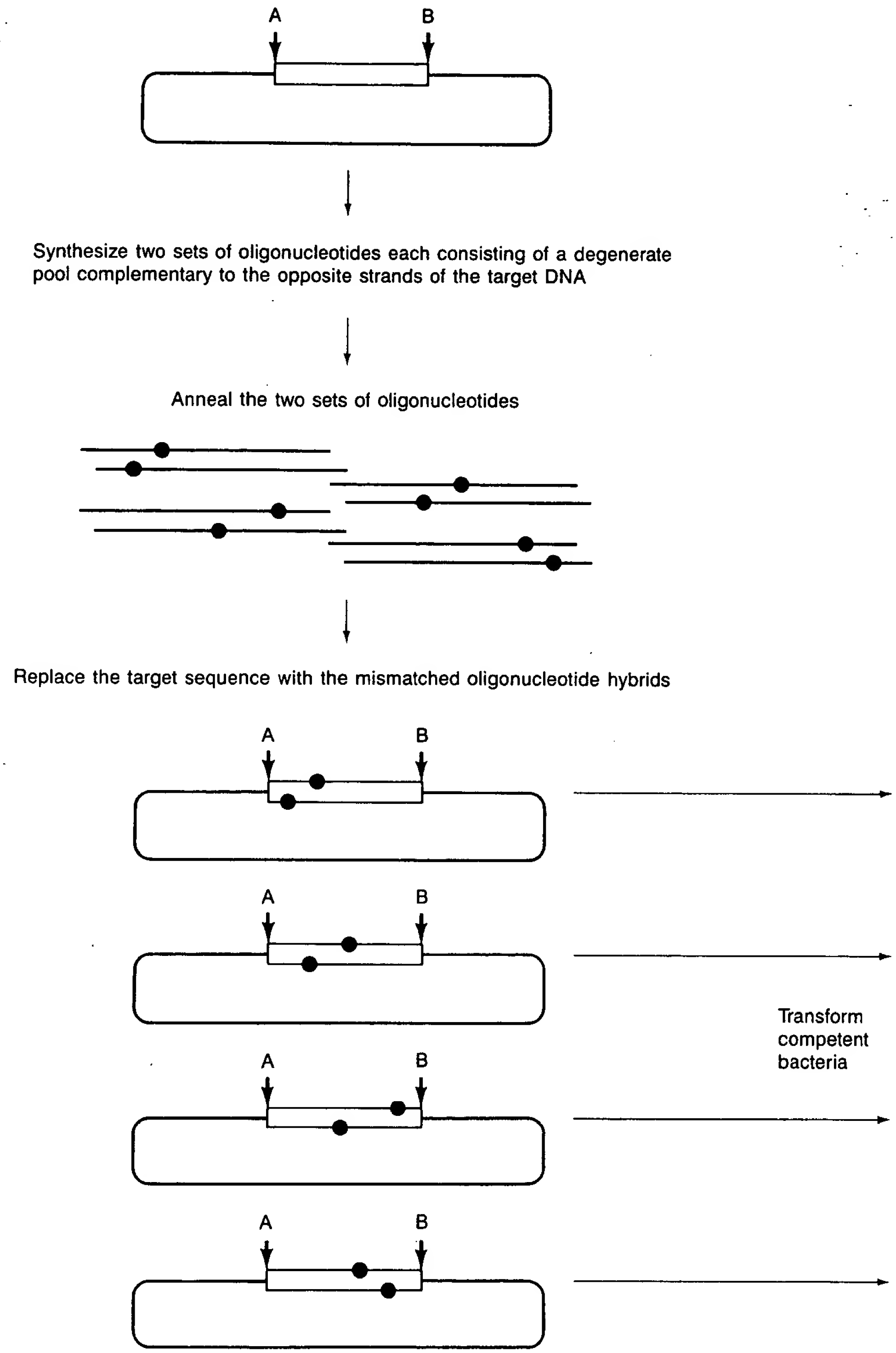
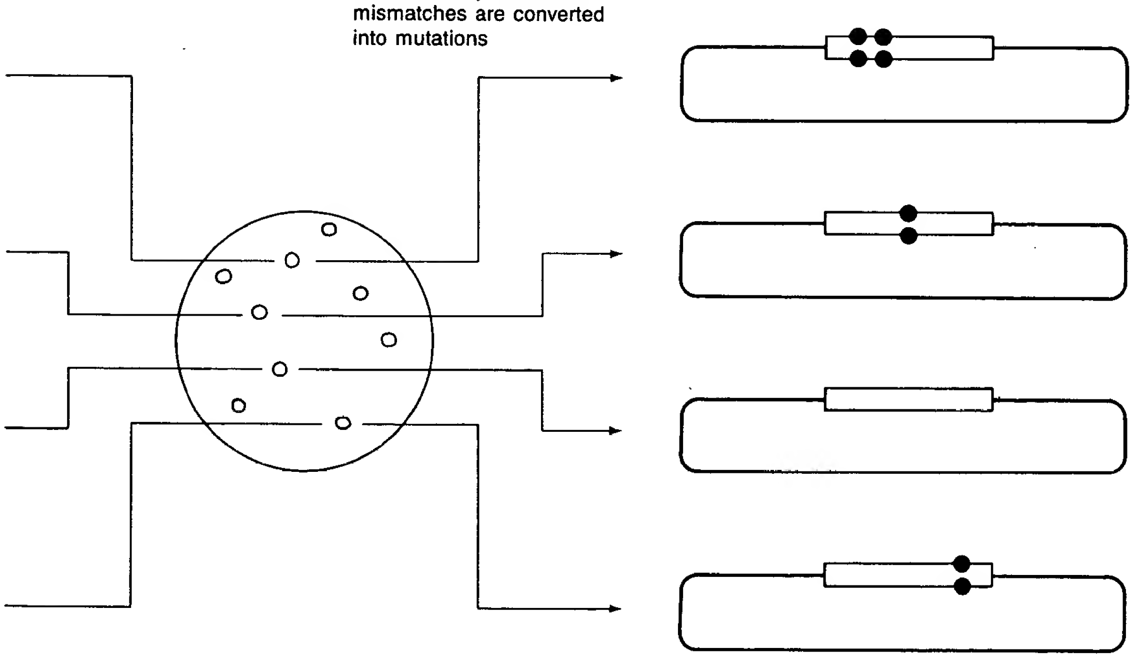


FIGURE 15.9B
Cassette mutagenesis using two complementary mixed-sequence oligonucleotides and repair of mismatches in vivo.

Mismatched repair in vivo generates plasmids in which approximately 50% of the mismatches are converted into mutations



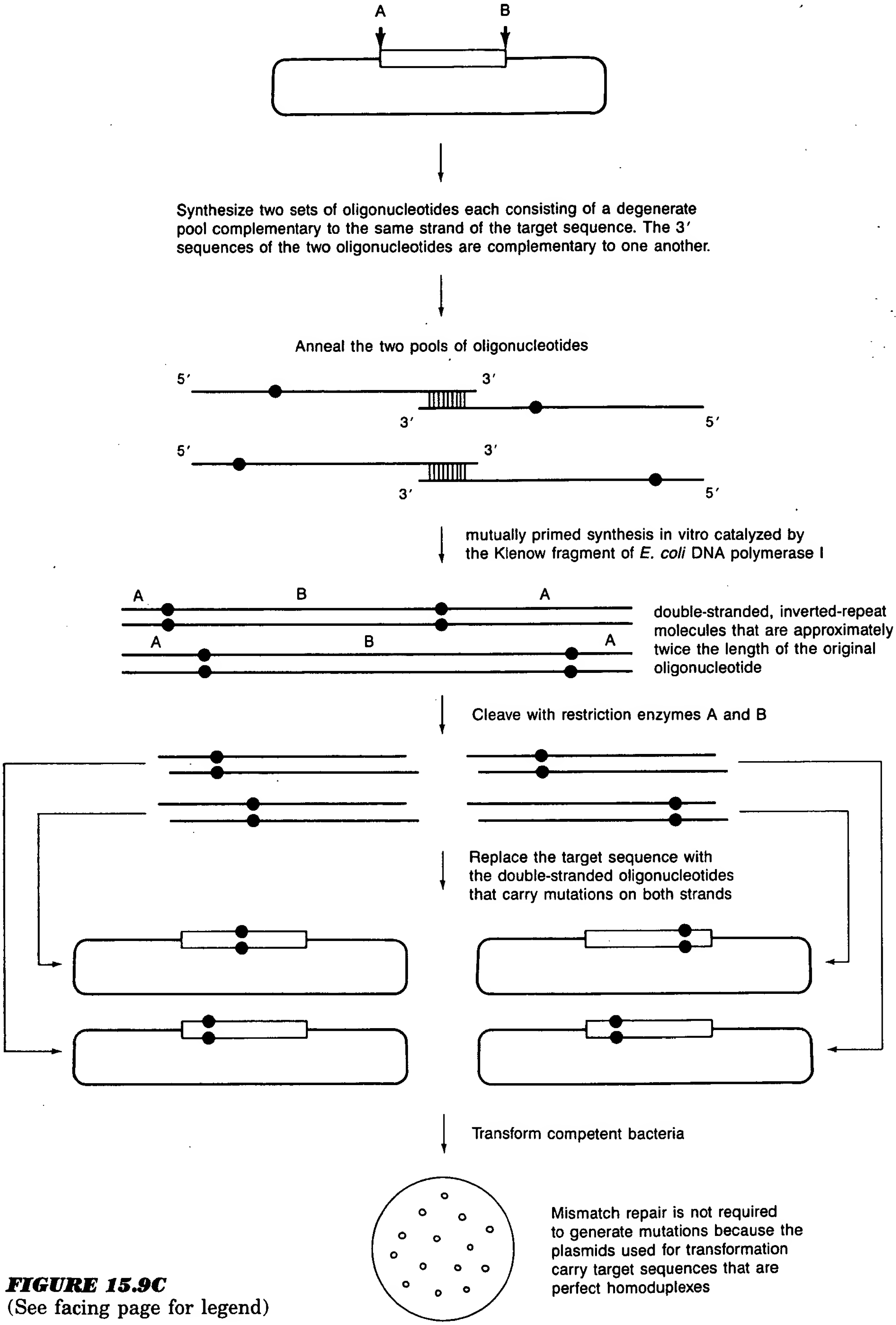


FIGURE 15.9C
(See facing page for legend)

mutants because of correction to wild-type sequences. Finally, because segregation is not required, primary bacterial transformants contain pure plasmid populations that can be analyzed directly. For these reasons, this is currently the method of choice for creating mutations at many sites within a defined region of DNA.

3. Ideally, each member of a degenerate pool of oligonucleotides should contain one nucleotide change per target sequence. In practice, however, because the oligonucleotide pools are generated in a mixed synthetic reaction, the best that can be achieved is an *average* of one altered base per target sequence. At each cycle, therefore, there is a chance that either a normal or an altered base will be incorporated into a growing oligonucleotide chain. The mutation frequency at any given site depends on the relative concentrations of the different nucleotide precursors that are provided at a particular cycle in the synthetic reaction.

   The fraction of oligonucleotides that contain nucleotide changes follows a binomial distribution that can be predicted from the following equation (McNeil and Smith 1985; Makris et al. 1988):

   $$F(P) = n!P^{n-r}(1-P)^r/(n-r)!r!$$

   where $F(P)$ is the fraction of the population whose sequence contains r random base changes over a target sequence of n consecutive bases, P is the probability of any given nucleotide being unchanged, and $(1-P)$ is the probability of any given nucleotide being changed. For example, when the length of the target sequence is 20 and the mixture of precursors supplied at every cycle contains 95% of the "normal" nucleotides and 5% of the "altered" nucleotides, the fraction of oligonucleotides that contain one altered nucleotide ($r = 1$) can be calculated as follows:

   $$\begin{aligned} F(P) &= 20!(0.95)^{19}(0.05)^1/19!1! \\ &= 0.38 \end{aligned}$$

   Similarly, 36% of the oligonucleotides in the pool will contain no alteration in nucleotide sequence; 19% of the oligonucleotides will contain two changes, and 7% will contain more than two changes.

4. The types of mutations created by degenerate pools of oligonucleotides depend on the precursors that are supplied at each round of the synthetic cycle. In the example discussed above, not more than 5% of the precursors provided at each round of synthesis can contain "abnormal" nucleotides. Within this 5%, however, the ratio of the three abnormal bases can be altered according to the needs of the particular experiment. Because transversions are usually more useful than transitions, many workers increase the proportion of abnormal bases that will generate transversions at the expense of abnormal bases that will cause transitions.

5. The termini of the oligonucleotides should not be mutagenized because they will be needed to insert the cassette into the appropriate plasmid. If

FIGURE 15.9C
Cassette mutagenesis using two partially overlapping mixed-sequence oligonucleotides and complementary strand synthesis in vitro.

cohesive termini are to be generated by cleaving the double-stranded cassettes with restriction enzymes, three extra nucleotides should be added to each end of the mutagenic oligonucleotide. These extensions increase the efficiency of digestion with restriction enzymes.

6. The frequency with which mutants are obtained at any particular position decreases as the length of the mutagenic oligonucleotides in the degenerate pool increases. Because individual mutants are recovered by random sampling, it is improbable that all possible mutations will be isolated when the size of the potential pool is large. Under these circumstances, "missing" clones that carry particularly interesting mutations can usually be identified by hybridization to specifically designed oligonucleotide probes.

Finally, it is worth remembering that oligonucleotide-mediated mutagenesis is not the only method that can be used to saturate segments of cloned DNA with mutations. Several of the other techniques that are available are discussed below.

Treatment of Double-stranded DNA with Chemical Mutagens

The simplest method of localized random mutagenesis is to react a short fragment of double-stranded DNA with a chemical mutagen such as nitrous acid or hydroxylamine and to clone the population of mutagenized fragments into a recombinant plasmid that carries the remainder of the wild-type gene. Recombinant plasmids carrying mutations that generate a novel phenotype can be recognized by appropriate tests. For example, a temperature-sensitive mutation constructed in a gene coding for a mammalian protein might be recognized by immunofluorescent staining of mammalian cells that had been transfected with the appropriate plasmid incubated at permissive and non-permissive temperatures. Recombinant plasmids carrying a mutation that does not give rise to an easily assayed phenotype must be identified by DNA sequencing of random clones. Unfortunately, the frequency at which mutants are recovered by this method is unacceptedly low (Chu et al. 1979; Solnick 1981; Busby et al. 1982; Kadonaga and Knowles 1985). Furthermore, because chemical mutagens react with bases in double-stranded DNA in a highly specific manner, only a limited spectrum of mutations is recovered. For these reasons, this method is no longer in widespread use.

Treatment of Single-stranded DNA with Sodium Bisulfite

In the original descriptions of this protocol, circular double-stranded plasmid DNA was nicked at a random site with pancreatic DNAase I in the presence of ethidium bromide (Greenfield et al. 1975; Shortle and Botstein 1983). The nick was then converted to a gap by digestion with exonuclease III, and the resulting gapped double-stranded molecule was exposed at slightly acid pH to sodium bisulfite (1–3 M), which caused deamination of cytosine to uracil. After transformation of bacteria, replication of the mutagenized DNA led to replacement of the original C:G base pair with a T:A base pair. Recently, the efficiency of this type of mutagenesis has been improved by carrying out deamination on gapped duplexes of bacteriophage M13 recombinants in which the target DNA is exposed in a single-stranded form (Pine and Huang 1987). After mutagenesis, the DNA is transfected into an *ung*$^-$ strain of *E. coli* that is unable to remove the newly generated uracil residues. Although the procedure results in highly efficient mutagenesis of a defined segment of DNA, it generates only transition mutations in which a purine replaces a purine on one strand of DNA and a pyrimidine replaces a pyrimidine on the other. Unfortunately, mutations of this type generally result in conservative substitutions of amino acids. Thus, the range of mutants that are obtained is often too narrow to allow a comprehensive analysis of a particular segment of a protein (Shortle and Nathans 1978; DiMaio and Nathans 1980; Peden and Nathans 1982).

Treatment of Single-stranded DNA with Chemicals That Damage All Four Bases

In this method (Myers et al. 1985a), single-stranded DNA of a recombinant M13 bacteriophage is exposed under defined conditions to chemicals (nitrous acid, formic acid, and hydrazine) that modify bases in single-stranded DNA without breaking the phosphodiester backbone (see Chapter 13). After removal of the chemicals, a universal sequencing primer and avian reverse transcriptase are used to synthesize the complementary strand of DNA. When the polymerase encounters damaged bases in the template strand, it incorporates nucleotides essentially at random. Because all possible nucleotides can be incorporated at a single position, there is a 75% probability of mutation at every site of damage. Furthermore, because tranversions are generated twice as frequently as transitions, the resulting mutations generate proteins with a wide spectrum of amino acid changes. After the extension reaction is completed, the double-stranded target fragment is excised and recloned into an appropriate vector. Mutants can be identified directly by DNA sequencing of random clones.

The major problem with this method is the frequency with which useful mutations can be isolated. To prevent the formation of unacceptable numbers of multiple mutants, it is necessary to limit carefully the length of time the single-stranded DNA is exposed to damaging chemicals. However, this means that many of the template strands escape modification altogether. Therefore, the best that can be achieved by this method is a frequency of single mutations of 10–15%. This problem can sometimes be alleviated by using denaturing gradient gel electrophoresis to purify fragments of DNA that carry mutations (Myers et al. 1985a,b). However, this technique is by no means simple, and it requires the attachment of the mutagenized DNA to special vectors equipped with GC clamps (Myers et al. 1985c). Because of these problems, this method of mutagenesis has so far not found widespread acceptance.

Misincorporation of Nucleotides by DNA Polymerases

Point mutations can be introduced into double-stranded DNA by incorporating base analogs with various types of DNA polymerases. For example, Shortle and his coworkers (Shortle et al. 1982; Shortle and Lin 1985) incubated gapped DNA in the presence of *E. coli* DNA polymerase I and only one of the four α-thiophosphate dNTPs. Thiophosphate dNTPs are efficiently incorporated by the polymerase but are not effectively removed by its $3' \rightarrow 5'$ editing function. The incorrect base is thus incorporated at a high frequency, and the remainder of the gap is then filled in a second polymerization reaction carried out in the presence of all four of the normal dNTPs. All types of base substitutions have been obtained with this method using each of the four α-thiophosphate dNTPs in separate repair reactions.

Another misincorporation method uses AMV reverse transcriptase, which is deficient in a $3' \rightarrow 5'$ exonuclease activity (Zakour and Loeb 1982). In this case, conventional dNTPs are used to synthesize DNA from an upstream primer. Base analogs are then incorporated in the region of interest.

The major problem of these and other misincorporation methods is the difficulty in creating populations of template molecules in which the 3′ hydroxyl terminus of the growing strand is located at random positions throughout the region of interest. Although this can in theory be achieved by a number of different methods (e.g., controlled nick translation with *E. coli* DNA polymerase I or controlled digestion of double-stranded DNA with exonuclease III), the routine generation of large numbers of mutations at random sites has proved to be difficult in practice. Success requires careful characterization of the reagents involved, meticulous establishment of optimal reaction conditions, and many trial experiments.

In summary, whereas methods (discussed earlier in this chapter) to introduce single mutations in cloned DNA are now well-established, techniques to saturate defined regions with mutations are less satisfactory. Using chemical mutagenesis, the rate of production of single mutations is low and/or the mutations themselves are of limited interest. Using misincorporation of base analogs, it is difficult to direct the mutations to the region of interest. However, it seems likely that at least some of these problems will be solved during the next few years, for example, by incorporating base changes into DNA synthesized in polymerase chain reactions or by advances in DNA chemistry that will facilitate the synthesis of mutagenized DNAs of extended length. Until then, we recommend using degenerate pools of synthetic oligonucleotides. In contrast to the other methods, the mutations can be precisely designed by the experimenter and can be focused in a defined region of DNA. Even with these limitations, the amount of work involved in isolating and characterizing a comprehensive set of mutants remains very large. In this branch of molecular cloning, therefore, it is especially important to weigh the potential scientific rewards against the commitment of time and personnel that the project will certainly consume.